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(54) Title: TECHNETIUM-99m LABELED PEPTIDES FOR IMAGING

(57) Abstract

This invention relates to radiolabeled peptides and methods for producing such peptides. Specifically, the invention relates to peptides, methods and kits for making such peptides, and methods for using such peptides to image sites in a mammalian body labeled with technetium-99m (Tc-99m) *via* a radiolabel-binding moiety which forms a neutral complex with Tc-99m.

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# TECHNETIUM-99m LABELED PEPTIDES FOR IMAGING

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

This invention relates to radiodiagnostic reagents and peptides, and methods for producing labeled radiodiagnostic agents. Specifically, the invention relates to peptides, methods and kits for making such peptides, and methods for using such peptides to image sites in a mammalian body labeled with technetium-99m (Tc-99m) *via* a radiolabel-binding moiety which forms a neutral complex with Tc-99m.

### 2. Description of the Prior Art

In the field of nuclear medicine, certain pathological conditions are localized, or their extent is assessed, by detecting the distribution of small quantities of internally-administered radioactively labeled tracer compounds (called radiotracers or radiopharmaceuticals). Methods for detecting these radiopharmaceuticals are known generally as imaging or radioimaging methods.

In radioimaging, the radiolabel is a gamma-radiation emitting radionuclide and the radiotracer is located using a gamma-radiation detecting camera (this process is often referred to as gamma scintigraphy). The imaged site is detectable because the radiotracer is chosen either to localize at a pathological site (termed positive contrast) or, alternatively, the radiotracer is chosen specifically not to localize at such pathological sites (termed negative contrast).

A number of factors must be considered for optimal radioimaging in humans. To maximize the efficiency of detection, a radionuclide that emits gamma energy in the 100 to 200 keV range is preferred. To minimize the absorbed radiation dose to the patient, the physical half-life of the radionuclide should be as short as the imaging procedure will allow. To allow for examinations to be performed on any day and at any time of the day, it is advantageous to have a source of the radionuclide always available at the clinical site.

A variety of radionuclides are known to be useful for radioimaging, including $^{67}Ga$, $^{99m}Tc$ (Tc-99m), $^{111}In$, $^{123}I$, $^{125}I$, $^{169}Yb$ or $^{186}Re$. Tc-99m is a preferred radionuclide because it emits gamma radiation at 140 keV, it has a physical half-life of 6 hours, and it is readily available on-site using a molybdenum-99/technetium-99m generator.

The sensitivity of imaging methods using radioactively-labeled peptides is much higher than other radiopharmaceuticals known in the art, since the specific binding of the radioactive peptide concentrates the radioactive signal over the area of interest. Small synthetic peptides that bind specifically to targets of interest may be advantageously used as the basis for radiotracers. This is because: 1. they may be synthesized chemically (as opposed to requiring their production in a biological system such as bacteria or mammalian cells, or their isolation from a biologically-derived substance such as a fragment of a protein); 2. they are small, hence non-target bound radiotracer is rapidly eliminated from the body, thereby reducing background (non-target) radioactivity and allowing good definition of the target; and 3. small peptides may be readily manipulated chemically to optimize their affinity for a particular binding site.

Small readily synthesized labeled peptide molecules are preferred as routinely-used radiopharmaceuticals. There is clearly a need for small synthetic labeled peptides that can be directly injected into a patient and will image pathological sites by localizing at such sites. Tc-99m labeled small synthetic peptides offer clear advantages as radiotracers for gamma scintigraphy, due to the properties of Tc-99m as a radionuclide for imaging and the utility of specific-binding small synthetic peptides as radiotracer molecules.

Radiolabeled peptides have been reported in the prior art.

Ege *et al.*, U.S. Patent No. 4,832,940 teach radiolabeled peptides for imaging localized T-lymphocytes.

Olexa *et al.*, 1982, European Patent Application No. 823017009 disclose a pharmaceutically acceptable radiolabeled peptide selected from Fragment $E_1$ isolated from cross-linked fibrin, Fragment $E_2$ isolated from cross-linked fibrin,

and peptides having an amino acid sequence intermediate between Fragments $E_1$ and $E_2$.

Ranby *et al.*, 1988, PCT/US88/02276 disclose a method for detecting fibrin deposits in an animal comprising covalently binding a radiolabeled compound to fibrin.

Hadley *et al.*, 1988, PCT/US88/03318 disclose a method for detecting a fibrin-platelet clot *in vivo* comprising the steps of (a) administering to a patient a labeled attenuated thrombolytic protein, wherein the label is selectively attached to a portion of the thrombolytic protein other than the fibrin binding domain; and (b) detecting the pattern of distribution of the labeled thrombolytic protein in the patient.

Lees *et al.*, 1989, PCT/US89/01854 teach radiolabeled peptides for arterial imaging.

Sobel, 1989, PCT/US89/02656 discloses a method to locate the position of one or more thrombi in an animal using radiolabeled, enzymatically inactive tissue plasminogen activator.

Stuttle, 1990, PCT/GB90/00933 discloses radioactively labeled peptides containing from 3 to 10 amino acids comprising the sequence arginine-glycine-aspartic acid (RGD), capable of binding to an RGD binding site *in vivo*.

Maraganore *et al.*, 1991, PCT/US90/04642 disclose a radiolabeled thrombus inhibitor comprising (a) a inhibitor moiety; (b) a linker moiety; and (c) and anion binding site moiety.

Rodwell *et al.*, 1991, PCT/US91/03116 disclose conjugates of "molecular recognition units" with "effector domains".

Tubis *et al.*, 1968, Int. J. Appl. Rad. Isot. 19: 835-840 describe labeling a peptide with technetium-99m.

Sundrehagen, 1983, Int. J. Appl. Rad. Isot. 34: 1003 describes labeling polypeptides with technetium-99m.

The use of chelating agents for radiolabeling polypeptides, and methods for labeling peptides and polypeptides with Tc-99m are known in the prior art and are disclosed in co-pending U.S. Patent Applications Serial Nos.

07/653,012 and 07/807,062, which are hereby incorporated by reference.

Although optimal for radioimaging, the chemistry of Tc-99m has not been as thoroughly studied as the chemistry of other elements and for this reason methods of radiolabeling with technetium are not abundant. Tc-99m is normally obtained as Tc-99m pertechnetate ($TcO_4^-$; technetium in the +7 oxidation state), usually from a molybdenum-99/technetium-99m generator. However, pertechnetate does not bind well to other compounds. Therefore, in order to radiolabel a peptide, Tc-99m pertechnetate must be converted to another form. Since technetium does not form a stable ion in aqueous solution, it must be held in such solutions in the form of a coordination complex that has sufficient kinetic and thermodynamic stability to prevent decomposition and resulting conversion of Tc-99m either to insoluble technetium dioxide or back to pertechnetate.

Such coordination complexes of Tc-99m (in the +1 to +6 oxidation states) are known. However, many of these complexes are inappropriate for radiolabeling due to the molecular geometry of the coordination complex. For the purpose of radiolabeling, it is particularly advantageous for the coordination complex to be formed as a chelate in which all of the donor groups surrounding the technetium ion are provided by a single chelating ligand. This allows the chelated Tc-99m to be covalently bound to a peptide through a single linker between the chelator and the peptide.

These ligands are sometimes referred to as bifunctional chelating agents having a chelating portion and a linking portion. Such compounds are known in the prior art.

Byrne *et al.*, U.S. Patent No. 4,434,151 describe homocysteine thiolactone-derived bifunctional chelating agents that can couple radionuclides to terminal amino-containing compounds that are capable of localizing in an organ or tissue to be imaged.

Fritzberg, U.S. Patent No. 4,444,690 describes a series of technetium-chelating agents based on 2,3-bis(mercaptoacetamido) propanoate.

Byrne *et al.*, U.S. Patent Nos. 4,571,430 describe novel homocysteine

thiolactone bifunctional chelating agents for chelating radionuclides that can couple radionuclides to terminal amino-containing compounds that are capable of localizing in an organ or tissue to be imaged.

Byrne *et al.*, U.S. Patent Nos. 4,575,556 describe novel homocysteine thiolactone bifunctional chelating agents for chelating radionuclides that can couple radionuclides to terminal amino-containing compounds that are capable of localizing in an organ or tissue to be imaged.

Davison *et al.*, U.S. Patent No. 4,673,562 describe technetium chelating complexes of bisamido-bisthio-ligands and salts thereof, used primarily as renal function monitoring agents.

Nicolotti *et al.*, U.S. Patent No. 4,861,869 describe bifunctional coupling agents useful in forming conjugates with biological molecules such as antibodies.

Fritzberg *et al.*, U.S. Patent 4,965,392 describe various S-protected mercaptoacetylglycylglycine-based chelators for labeling proteins.

Fritzberg *et al.*, European Patent Application No. 86100360.6 describe dithiol, diamino, or diamidocarboxylic acid or amine complexes useful for making technetium-labeled imaging agents.

Dean *et al.*, 1989, PCT/US89/02634 describe bifunctional coupling agents for radiolabeling proteins and peptides.

Flanagan *et al.*, European Patent Application No. 90306428.5 disclose Tc-99m labeling of synthetic peptide fragments *via* a set of organic chelating molecules.

Albert *et al.*, European Patent Application No. WO 91/01144 disclose radioimaging using radiolabeled peptides related to growth factors, hormones, interferons and cytokines and comprised of a specific recognition peptide covalently linked to a radionuclide chelating group.

Dean, co-pending U.S. Patent Application Serial No. 07/653,012 teaches reagents and methods for preparing peptides comprising a Tc-99m chelating group covalently linked to a specific binding peptide for radioimaging *in vivo*, and is hereby incorporated by reference.

(It is noted that all of these procedures would be expected to form anionic complexes of technetium in the +5 oxidation state.)

Baidoo & Lever, 1990, Bioconjugate Chem. 1: 132-137 describe a method for labeling biomolecules using a bisamine bisthiol group that gives a cationic technetium complex.

It is possible to radiolabel a peptide by simply adding a thiol-containing moiety such as cysteine or mercaptoacetic acid. Such procedures have been described in the prior art.

Schochat *et al.*, U.S. Patent No. 5,061,641 disclose direct radiolabeling of proteins comprised of at least one "pendent" sulfhydryl group.

Dean *et al.*, co-pending U.S. Patent Application 07/807,062 teach radiolabeling peptides *via* attached groups containing free thiols, and is incorporated herein by reference.

Goedemans *et al.*, PCT Application No. WO 89/07456 describe radiolabeling proteins using cyclic thiol compounds, particularly 2-iminothiolane and derivatives.

Thornback *et al.*, EPC Application No. 90402206.8 describe preparation and use of radiolabeled proteins or peptides using thiol-containing compounds, particularly 2-iminothiolane.

Stuttle, PCT Application No. WO 90/15818 describes Tc-99m labeling of RGD-containing oligopeptides.

Again it is noted that in all of these cases the expected Tc-99m labeled species would be an anionic complex.

The binding of certain peptides to their target entities is sensitive to charge modification of the peptide. Thus, it is disadvantageous in some cases to radiolabel a peptide with Tc-99m *via* a chelator that will form a charged Tc-99m complex. It is advantageous in certain cases to use a chelator that will form an electrically neutral or uncharged Tc-99m complex.

This invention provides chelators for Tc-99m which may be used to prepare Tc-99m labeled peptides in which the Tc-99m is held as a neutral chelate complex.

Some chelators said to form neutral Tc-99m complexes have been described in the prior art.

Burns *et al.*, 1985, European Patent Application 85104959.3 describe bisamine bisthiol compounds for making small neutral Tc-99m brain imaging agents.

Kung *et al.*, 1986, European Patent Application 86105920.2 describe bisamine bisthiol compounds for making small neutral Tc-99m imaging agents.

Bergstein *et al.*, 1988, European Patent Application 88102252.9 describe bisamine bisthiol compounds for making small neutral Tc-99m brain imaging agents.

Bryson *et al.*, 1988, Inorg. Chem. 27: 2154-2161 describe neutral complexes of technetium-99 which are unstable to excess ligand..

Misra *et al.*, 1989, Tet. Let. 30: 1885-1888 describe bisamine bisthiol compounds for radiolabeling purposes.

Bryson *et al.*, 1990, Inorg. Chem. 29: 2948-2951 describe chelators containing two amide groups, a thiol group and a substituted pyridine that may form neutral Tc-99 complexes.

Taylor *et al.*, 1990, J. Nucl. Med. 31: 885 (Abst) describe a neutral Tc-99m complex for brain imaging.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates hypercholsterolic and normal rabbit aortae stained with Sudan IV.

Figure 2 illustrates hypercholesterolic and normal rabbit aortae imaged *in vivo* with P215 for 2.5h

Figure 3 illustrates hypercholesterolic and normal rabbit aortae imaged *ex corpora* with P215.

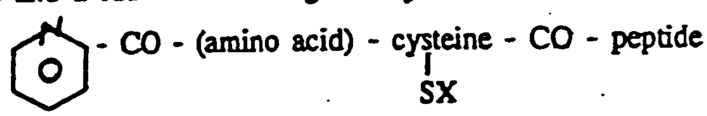
Figure 4 illustrates a thrombus imaged *in vivo* in a dog leg with Tc-99m labeled P357.

## SUMMARY OF THE INVENTION

The present invention provides scintigraphic imaging agents that are radioactively-labeled peptides. The radiolabeled peptides of the invention are comprised of peptides that specifically bind to a target *in vivo* and are covalently linked to a radiolabel-binding moiety wherein the moiety binds a radioisotope. It is a particular advantage in the present invention that the complex of the radiolabel-binding moiety and the radiolabel is electrically neutral, thereby avoiding interference of the covalently linked radiolabeled complex with the specific binding properties of the specific binding peptide.

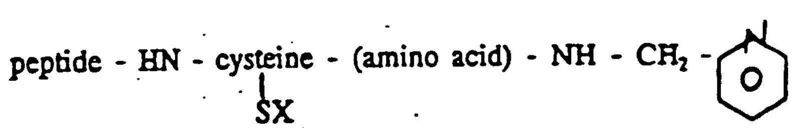
In a first aspect of the present invention, radiolabeled peptides are provided capable of imaging sites within a mammalian body. The peptides are comprised of a specific binding peptide having an amino acid sequence and a radiolabel-binding moiety covalently linked to the peptide. Further, the complex of the radiolabel-binding moiety and the radiolabel is electrically neutral. In a preferred embodiment, the peptide is covalently linked to the radiolabel-binding moiety *via* an amino acid, most preferably glycine. In another preferred embodiment, the radiolabel is technetium-99m.

In a second embodiment, the invention provides a radiolabeled peptide for imaging sites within a mammalian body, comprising a specific binding peptide and a radiolabel-binding moiety of formula:




[for purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolinic acid (Pic)-based moieties]

or



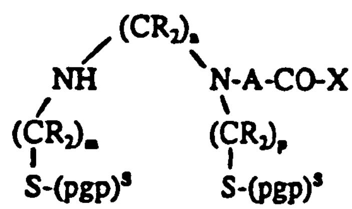

[for purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolylamine (Pica)-based moieties]

wherein X is H or a protecting group; (amino acid) is any amino acid; the radiolabel-binding moiety is covalently linked to the peptide and the complex

of the radiolabel-binding moiety and the radiolabel is electrically neutral. In a preferred embodiment, the amino acid is glycine and X is an acetamidomethyl protecting group. In additional preferred embodiments, the peptide is covalently linked to the radiolabel-binding moiety *via* an amino acid, most preferably glycine, and the radiolabel is technetium-99m.

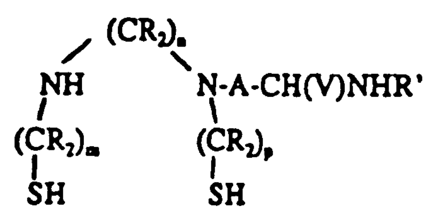
In yet another embodiment of the invention, a radiolabeled peptide is provided for imaging sites within a mammalian body, comprising a specific binding peptide and a bisamino bisthiol radiolabel-binding moiety covalently linked to the peptide. The bisamino bisthiol radiolabel-binding moiety in this embodiment of the invention has a formula selected from the group consisting of:

I.



wherein each R can be independently H, $CH_3$ or $C_2H_5$; each $(pgp)^s$ can be independently a thiol protecting group or H; m, n and p are independently 2 or 3; A is linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof; and X is peptide;

II.



wherein each R is independently H, $CH_3$ or $C_2H_5$; m, n and p are independently 2 or 3; A is linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof; V is H or CO-peptide; R' is H or peptide; provided that when V is H, R' is peptide and when R' is H, V is peptide. [For purposes of this invention, radiolabel-binding moieties having these structures will be referred to as "BAT" moieties]. In a preferred

embodiment, the peptide is covalently linked to the radiolabel-binding moiety *via* an amino acid, most preferably glycine, and the radiolabel is technetium-99m.

In preferred embodiments of the aforementioned aspects of this invention, the specific binding compound is a peptide is comprised of between 3 and 100 amino acids. The most preferred embodiment of the radiolabel is technetium-99m.

Specific-binding peptides provided by the invention include but are not limited to peptides having the following sequences:

formyl-MLF
$(VGVAPG)_3$amide
$(VPGVG)_4$amide
RALVDTLKFVTQAEGAKamide
RALVDTEFKVKQEAGAKamide
PLARITLPDFRLPEIAIPamide
GQQHHLGGAKAGDV
PLYKKIIKKLLES
LRALVDTLKamide
GGGLRALVDTLKamide
GGGLRALVDTLKFVTQAEGAKamide
GGGRALVDTLKALVDTLamide
GHRPLDKKREEAPSLRPAPPPISGGGYR
PSPSPIHPAHHKRDRRQamide
GGGF$_D$.Cpa.YW$_D$KTFTamide
GGCNP.Apc.GDC
S——S
$[SYNRGDSTC]_3$-TSEA
GGGLRALVDTLKamide
GCGGGLRALVDTLKamide
GCYRALVDTLKFVTQAEGAKamide
GC$(VGVAPG)_3$amide

The reagents of the invention may be formed wherein the specific binding compounds or the radiolabel-binding moieties are covalently linked to a polyvalent linking moiety. Polyvalent linking moieties of the invention are comprised of at least 2 identical linker functional groups capable of covalently bonding to specific binding compounds or radiolabel-binding moieties. Preferred linker functional groups are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups. In preferred

embodiments, the polyvalent linking moieties are comprised of *bis*-succinimdylmethylether (BSME), 4-(2,2-dimethylacetyl)benzoic acid (DMAB), *tris*(succinimidylethyl)amine (TSEA) and *N*-[2-(*N'*,*N'*-*bis*(2-succinimidoethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide (BAT-BS).

The invention also comprises complexes of the peptides of the invention with Tc-99m and methods for radiolabeling the peptides of the invention with Tc-99m. Radiolabeled complexes provided by the invention are formed by reacting the peptides of the invention with Tc-99m in the presence of a reducing agent. Preferred reducing agents include but are not limited to dithionite ion, stannous ion, and ferrous ion. Complexes of the invention are also formed by labeling the peptides of the invention with Tc-99m by ligand exchange of a prereduced Tc-99m complex as provided herein.

The invention also provides kits for preparing the peptides of the invention radiolabeled with Tc-99m. Kits for labeling the peptide of the invention with Tc-99m are comprised of a sealed vial containing a predetermined quantity of a peptide of the invention and a sufficient amount of reducing agent to label the peptide with Tc-99m.

This invention provides methods for preparing peptides of the invention by chemical synthesis *in vitro*. In a preferred embodiment, peptides are synthesized by solid phase peptide synthesis.

This invention provides methods for using Tc-99m labeled peptides for imaging a site within a mammalian body by obtaining *in vivo* gamma scintigraphic images. These methods comprise administering an effective diagnostic amount of a Tc-99m radiolabeled peptide of the invention and detecting the gamma radiation emitted by the Tc-99m localized at the site within the mammalian body.

Compositions of matter comprising radiolabel-binding moieties that form an electrically neutral complex with a radioisotope are also provided by the invention. In a preferred embodiment, the radioisotope is Tc-99m. Additional preferred embodiments include bisamine, bisthiol derivatives and picolinic acid

and picolylamine derivatives described herein.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides Tc-99m labeled peptides for imaging target sites within a mammalian body comprising an amino acid sequence covalently linked to a radiolabel-binding moiety wherein the radiolabel-binding moiety binds a radioisotope and forms an electrically neutral complex.

Labeling with Tc-99m is an advantage of the present invention because the nuclear and radioactive properties of this isotope make it an ideal scintigraphic imaging agent. This isotope has a single photon energy of 140 keV and a radioactive half-life of about 6 hours, and is readily available from a $^{99}Mo$-$^{99m}Tc$ generator. Other radionuclides known in the prior art have effective half-lives which are much longer (*for example*, $^{111}In$, which has a half-life of 67.4 h) or are toxic (*for example*, $^{125}I$).

In the radiolabel binding moieties and peptides covalently linked to such moieties that contain a thiol covalently linked to a thiol protecting groups [$(pgp)^S$] provided by the invention, the thiol-protecting groups may be the same or different and may be but are not limited to:

-$CH_2$-aryl (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-CH-$(aryl)_2$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-C-$(aryl)_3$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-$CH_2$-(4-methoxyphenyl);

-CH-(4-pyridyl)$(phenyl)_2$;

-$C(CH_3)_3$

-9-phenylfluorenyl;

-$CH_2$NHCOR (R is unsubstituted or substituted alkyl or aryl);

-$CH_2$-NHCOOR (R is unsubstituted or substituted alkyl or aryl);

-CONHR (R is unsubstituted or substituted alkyl or aryl);

$-CH_2-S-CH_2$-phenyl

Preferred protecting groups have the formula $-CH_2-NHCOR$ wherein R is a lower alkyl having 1 and 8 carbon atoms, phenyl or phenyl-substituted with lower alkyl, hydroxyl, lower alkoxy, carboxy, or lower alkoxycarbonyl. The most preferred protecting group is an acetamidomethyl group.

Each specific-binding peptide-containing embodiment of the invention is comprised of a sequence of amino acids. The term amino acid as used in this invention is intended to include all L- and D- amino acids, naturally occurring and otherwise.

Peptides of the present invention can be chemically synthesized *in vitro*. Peptides of the present invention can generally advantageously be prepared on an amino acid synthesizer. The peptides of this invention can be synthesized wherein the radiolabel-binding moiety is covalently linked to the peptide during chemical synthesis *in vitro*, using techniques well known to those with skill in the art. Such peptides covalently-linked to the radiolabel-binding moiety during synthesis are advantageous because specific sites of covalent linkage can be determined.

Radiolabel binding moieties of the invention may be introduced into the target specific peptide during peptide synthesis. For embodiments [*e.g.*, Pic-Gly-Cys(protecting group)-] comprising picolinic acid (Pic-), the radiolabel-binding moiety can be synthesized as the last (i.e., amino-terminal) residue in the synthesis. In addition, the picolinic acid-containing radiolabel-binding moiety may be covalently linked to the $\epsilon$-amino group of lysine to give, for example, $\alpha$N(Fmoc)-Lys-$\epsilon$N[Pic-Gly-Cys(protecting group)], which may be incorporated at any position in the peptide chain. This sequence is particularly advantageous as it affords an easy mode of incorporation into the target binding peptide.

Similarly, the picolylamine (Pica)-containing radiolabel-binding moiety [-Cys(protecting group)-Gly-Pica] can be prepared during peptide synthesis by including the sequence [-Cys(protecting group)-Gly-] at the carboxyl terminus of the peptide chain. Following cleavage of the peptide from the resin the

carboxyl terminus of the peptide is activated and coupled to picolylamine. This synthetic route requires that reactive side-chain functionalities remain masked (protected) and do not react during the conjugation of the picolylamine.

Examples of small synthetic peptides containing the Pic-Gly-Cys- and -Cys-Gly-Pica chelators are provided in the Examples hereinbelow. This invention provides for the incorporation of these chelators into virtually any peptide, resulting in a radiolabeled peptide having Tc-99m held as neutral complex.

This invention also provides specific-binding small synthetic peptides which incorporate bisamine bisthiol (BAT) chelators which may be labeled with Tc-99m, resulting in a radiolabeled peptide having Tc-99m held as neutral complex. Examples of small synthetic peptides containing these BAT chelators as radiolabel-binding moiety are provided in the Examples hereinbelow.

In forming a complex of radioactive technetium with the peptides of this invention, the technetium complex, preferably a salt of Tc-99m pertechnetate, is reacted with the peptides of this invention in the presence of a reducing agent. Preferred reducing agents are dithionite, stannous and ferrous ions; the most preferred reducing agent is stannous chloride. In an additional preferred embodiment, the reducing agent is a solid-phase reducing agent. Complexes and means for preparing such complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of a peptide of the invention to be labeled and a sufficient amount of reducing agent to label the peptide with Tc-99m. Alternatively, the complex may be formed by reacting a peptide of this invention with a pre-formed labile complex of technetium and another compound known as a transfer ligand. This process is known as ligand exchange and is well known to those skilled in the art. The labile complex may be formed using such transfer ligands as tartrate, citrate, gluconate or mannitol, for example. Among the Tc-99m pertechnetate salts useful with the present invention are included the alkali metal salts such as the sodium salt, or ammonium salts or lower alkyl ammonium salts.

In a preferred embodiment of the invention, a kit for preparing

technetium-labeled peptides is provided. The peptides of the invention can be chemically synthesized using methods and means well-known to those with skill in the art and described hereinbelow. Peptides thus prepared are comprised of between 3 and 100 amino acid residues, and are covalently linked to a radiolabel-binding moiety wherein the radiolabel-binding moiety binds a radioisotope. An appropriate amount of the peptide is introduced into a vial containing a reducing agent, such as stannous chloride or a solid-phase reducing agent, in an amount sufficient to label the peptide with Tc-99m. An appropriate amount of a transfer ligand as described (such as tartrate, citrate, gluconate or mannitol, for example) can also be included. Technetium-labeled peptides according to the present invention can be prepared by the addition of an appropriate amount of Tc-99m or Tc-99m complex into the vials and reaction under conditions described in Example 3 hereinbelow.

Radioactively labeled peptides provided by the present invention are provided having a suitable amount of radioactivity. In forming Tc-99m radioactive complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 millicurie (mCi) to 100 mCi per mL.

Technetium-labeled peptides provided by the present invention can be used for visualizing sites in a mammalian body. In accordance with this invention, the technetium-labeled peptides or neutral complexes thereof are administered in a single unit injectable dose. Any of the common carriers known to those with skill in the art, such as sterile saline solution or plasma, can be utilized after radiolabeling for preparing the injectable solution to diagnostically image various organs, tumors and the like in accordance with this invention. Generally, the unit dose to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 mL to about 10 mL. After intravenous administration, imaging of the organ or tumor *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after the radiolabeled peptide is injected

into a patient. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos. Any conventional method of scintigraphic imaging for diagnostic purposes can be utilized in accordance with this invention.

The technetium-labeled peptides and complexes provided by the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Among the preferred media are normal saline and plasma.

The methods for making and labeling these compounds are more fully illustrated in the following Examples. These Examples illustrate certain aspects of the above-described method and advantageous results. These Examples are shown by way of illustration and not by way of limitation.

## EXAMPLE 1

### <u>Synthesis of BAT Chelators</u>

A. Synthesis of *N*-Boc-*N'*-(5-carboxypentyl)-*N*,*N'*-bis(2-methyl-2-triphenylmethylthiopropyl)ethylenediamine

a. <u>2-methyl-2-(triphenylmethylthio)propanal</u>

Triphenylmethylmercaptan (362.94 g, 1.31 mol, 100 mol%) dissolved in anhydrous THF (2 L) was cooled in an ice bath under argon. Sodium hydride (60% in oil; 54.39 g, 1.35 mol, 104 mol%) was added in portions over 20 min. 2-bromo-2-methylpropanal (206.06 g, 1.36 mol, 104 mol%; *see* Stevens & Gillis, 1957, J. Amer. Chem. Soc. <u>79</u>: 3448-51) was then added slowly over 20 min. The reaction mixture was allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with water (1 L) and extracted with diethyl ether (3x 1 L). The ether extracts were combined, washed with saturated NaCl solution (500 mL), dried over $Na_2SO_4$ and filtered. The solvent was removed under reduced pressure to afford a thick orange oil. The crude oil was dissolved in toluene (200 mL) and diluted to

2 L with hot hexanes. The mixture was filtered through a sintered glass funnel and cooled at -5°C for 12 hours. The white crystalline solid which formed was removed by filtration to afford 266.36 g (59% yield) of the title compound. The melting point of the resulting compound was determined to be 83-85°C. Nuclear magnetic resonance characterization experiments yielded the following molecular signature:

$^1$H NMR(300 MH$_z$, $CDCl_3$): $\delta$ 1.24(s, 6H, $2CH_3$), 7.2 - 7.35 (m, 9H), 7.59-7.62 (m,6H), 8.69 (s, H, -COH)

$^{13}$C NMR (75 MH$_z$, $CDCl_3$): $\delta$ 22.86, 55.66, 67.48, 126.85, 127.75, 129.72, 144.79, 197.31.

b. N,N'-bis(2-methyl-2-triphenylmethylthiopropyl)ethylenediamine.

Ethylenediamine (1.3 mL, 0.0194 mol, 100 mol%) was added to 2-methyl-2-(triphenylmethylthio)propanal (13.86 g, 0.0401 mol, 206 mol%) dissolved in methanol (40 mL) and anhydrous THF (40 mL) under argon, and the pH was adjusted to pH 6 by dropwise addition of acetic acid. The solution was stirred for 20 min at 20°C. Sodium cyanoborohydride (1.22 g, 0.0194 mol, 100 mol%) was added and the reaction was stirred at room temperature for 3 hours. Additional sodium cyanoborohydride (1.08 g) was added and the reaction was stirred at 20°C for 17 hours. A final portion of sodium cyanoborohydride (1.02 g) was added and the reaction heated at reflux under argon for 6 hours. The reaction was quenched with 0.5 M HCl (100 mL) and extracted with ethyl acetate (2x 100 mL). The organic extracts were combined, sequentially washed with 2 M NaOH (60 mL), saturated NaCl solution (60 mL), dried ($Na_2SO_4$), and filtered. The solvent was removed under reduced pressure to give 16.67 g of crude product which was crystallized from toluene/hexanes to afford 10.20 g (73% yield) of white crystals of the title compound. The melting point of the resulting compound was determined to be 83-86°C. FABMS analysis yielded an m/z of 721 (MH+). Nuclear magnetic resonance characterization experiments yielded the following molecular signature:

$^1$H NMR (300 MHz, $CDCl_3$): δ 1.12 (s, 12H, 4 $CH_3$), 1.64 (s, 4H, N-$CH_2$-C$(Me)_2$-S), 2.52 (s, 4H, N-$CH_2$-$CH_2$-N), 5.31 (S, 2H, 2-NH), 7.12-7.30 (m, 18H, Ar), 7.62-7.65 (m, 12H, Ar).

c. N-(5-carboethoxypentyl)-N,N'-bis(2-methyl-2-triphenylmethylthiopropyl)ethylenediamine

$K_2CO_3$ (1.92 g, 13.9 mmol, 100 mol%) was added to N,N'-bis(2-methyl-2-triphenylmethylthiopropyl)ethylenediamine (10.03 g, 13.9 mmol) in $CH_3CN$ (60 mL), followed by ethyl 5-bromovalerate (3.30 mL, 20.8 mmol, 150 mol%). The reaction was heated at reflux under argon overnight. The solution was then concentrated to a paste and partitioned between 0.25 M KOH (100 mL) and ethyl acetate (100 mL). The aqueous layer was extracted with ethyl acetate (1x 50 mL) and the combined ethyl acetate layers were washed with 50 mL water and NaCl solution (2x 50 mL), dried with $Na_2SO_4$ and concentrated to an orange oil. Purification by flash chromatography (300 g flash silica, 100% $CHCl_3$ to 5% MeOH/$CHCl_3$) gave pure title compound (7.75 g, 66% yield). FABMS analysis yielded an (MH+) of 849 (compared with a calculated molecular weight of 849.24 for the compound $C_{55}H_{64}N_2O_2S_2$).

d. *N*-Boc-*N'*-(5-carboxypentyl)-*N*,*N'*-bis(2-methyl-2-triphenylmethylthiopropyl)ethylenediamine

1M KOH (25 mL, 25.0 mmol, 274 mol%) was added to N-(5-carboethoxypentyl)-N,N'-bis(2-methyl-2-triphenylmethylthiopropyl) ethylenediamine (7.75 g, 9.13 mmol) in dioxane (200 mL), followed by water (250 mL). Dioxane was then added dropwise with stirring until a homogeneous solution was obtained. The reaction was heated at a slow reflux overnight. Most of the dioxane was removed by rotary evaporation and the pH of solution was adjusted to ~7-8 with 1 M $KH_2PO_4$ and saturated $NaHCO_3$. The solution was then extracted with ethyl acetate (3x 75 mL) and the combined organic layers were washed with NaCl solution (50 mL), dried with $Na_2SO_4$ and concentrated to a foam/solid (6.35 g, 85% yield).

To the crude product from the above reaction was added $(BOC)_2O$ (3.35

g, 15.4 mmol, 200 mol%), $CH_3CN$ (50 mL) and methylene chloride (50 mL), followed by triethylamine (1.0 mL, 7.2 mmol, 93 mol%). The reaction was stirred at room temperature under argon overnight. The reaction solution was then concentrated and partitioned between water (100 mL) and ethyl acetate (50 mL). The aqueous layer was extracted with ethyl acetate (1x 50 mL) and the combined ethyl acetate layers were washed with 5% citric acid and NaCl solution (50 mL each), then dried ($Na_2SO_4$) and concentrated to an orange oil. Purification by flash chromatography (200 g flash silica, 100% $CDCl_3$ to 5% methanol/chloroform) gave pure title compound (2.58 g, 36% yield). FABMS analysis gave an (MH+) of 921 (compared with the calculated value of 921.31 for the compound $C_{51}H_{64}N_2O_4S_2$).

**B. Synthesis of *N*-Boc-*N'*-(5-carboxypentyl)-*N,N'*-bis-[2-(4-methoxybenzylthio)-2-methylpropyl]ethylenediamine**

a. *N,N'*-bis-[2-(4-methoxybenzylthio)-2-methylpropyl]-ethylenediamine

A solution of *N,N'*-bis(2-mercapto-2-methylpropyl)ethylenediamine (11.23 g, 47.5 mmol; *see*, DiZio *et al.*, 1991, Bioconjugate Chem 2: 353 and Corbin *et al.*, 1976, J. Org. Chem. 41: 489) in methanol (500 mL) was cooled in ice/water bath and then saturated with gaseous ammonia over 45 min. To this was added 4-methoxybenzyl chloride (17.0 mL, 125 mmol, 264 mol%). The reaction was allowed to warm to room temperature overnight with stirring under argon. The solution was concentrated to a paste and then partitioned between diethyl ether (150 mL) and 0.5 M KOH (200 mL). The aqueous layer was further extracted with diethyl ether (2x 50 mL). The combined organic layers were washed with NaCl solution and concentrated to a clear colorless oil. The oil dissolved in diethyl ether (200 mL) and then acidified with 4.0 M HCl in dioxane until no further precipitation was seen. The white precipitate was collected by filtration and washed with diethyl ether. The white solid was recrystallized from hot water at a pH of ~2. The product was collected by filtration to afford 29.94 g as a mix of mono- and di- HCl salts. The HCl salts were partitioned between 1 M KOH (100 mL) and ethyl acetate (100 mL). The aqueous was extracted with ethyl acetate (2x 30 mL) and the

combined organic layers were washed with NaCl solution, dried with $Na_2SO_4$ and concentrated to give pure product as the free base as a light yellow oil (18.53 g, 82% yield). Nuclear magnetic resonance characterization experiments yielded the following molecular signature:

$^1$H NMR (300 MHz, $CDCL_3$): δ 7.25 (d, 4H, J=9), 6.83 (d, 4H, J=9), 3.78 (s, 6H), 3.67 (s, 4H), 2.63 (s, 4H), 2.56 (s, 4H), 1.34 (s, 12H).

b. *N*-(5-carboethoxypentyl)-*N,N'-bis*-[2-(4-methoxybenzylthio)-2-methylpropyl]ethylenediamine

To *N,N'-bis*-[2-(4-methoxybenzylthio)-2-methylpropyl]-ethylenediamine (4.13 g, 8.66 mmol) in $CH_3CN$ (50 mL) was added $K_2CO_3$ (1.21 g, 8.75 mmol, 101 mol%) followed by ethyl 5-bromovalerate (2.80 mL, 17.7 mmol, 204 mol%). The reaction was stirred at reflux overnight and was then concentrated to a paste in vacuo. The residue was partitioned between ethyl acetate (100 mL) and 0.5 M KOH (100 mL). The aqueous layer was extracted with ethyl acetate (1x 50 mL) and the combined organic layers were washed with NaCl solution (50 mL), dried with $Na_2SO_4$ and concentrated to a yellow oil (~6 g). Purification by normal-phase preparative HPLC (100% $CHCl_3$ to 5% methanol/chloroform over 25 min.) afforded pure title compound (1.759 g, 34% yield). FABMS analysis gave an (MH+) of 605 (compared with the value of 604.90 calculated for $C_{33}H_{52}N_2O_4S_2$). Nuclear magnetic resonance characterization experiments yielded the following molecular signature:

$^1$H NMR (300 mH$_z$, $CDCl_3$): δ 7.25 (d, 4H, J=8.5), 6.83 (d, 4H, J=8.5), 4.13 (q, 2H, J=7), 3.793 (s, 3H), 3.789 (s. 3H), 3.74 (s, 2H), 3.67 (s, 2H), 2.6 (m, 10H), 2.31 (t, 2H, J=7), 1.6 (m, 2H), 1.5 (m 2H), 1.34 (s 12H), 1.28 (t, 3H, J=7).

c. *N*-Boc-*N'*-(5-carboxypentyl)-*N,N'-bis*-[2-(4-methoxybenzylthio)-2-methylpropyl]ethylenediamine

To *N*-(5-carboethoxypentyl)-*N,N'-bis*-[2-(4-methoxybenzylthio)-2-methylpropyl]ethylenediamine (586 mg, 0.969 mmol) in THF (40 mL) was added water (30 mL) and 1 M KOH (2.5 mL, 2.5 mmol, 260 mol%). The

homogeneous solution was heated to a slow reflux overnight. The solution was then cooled to room temperature and the THF was removed under rotary evaporation. The residue was diluted to 50 mL with $H_2O$ and the pH was adjusted to ~2-3 with 1 M HCl. The solution was extracted with ethyl acetate (3x 30 mL) and the combined organic layers were washed with NaCl solution (50 mL), dried with $Na_2SO_4$ and concentrated to give crude acid (422 mg, 75% yield).

To the crude product from the above reaction was added $CH_3CN$ (40 mL) and $(BOC)_2O$ (240 mg, 1.10 mmol, 150 mol%) followed by triethylamine (0.200 mL, 1.43 mmol, 196 mol%). The homogenous solution stirred at room temperature overnight under argon. The solution was then concentrated to a paste and partitioned between ethyl acetate (25 mL) and 1 M $KH2PO_4$ (25 mL). The organic layer was washed with 5% citric acid (2x 25 mL) and NaCl solution (25 mL), dried with $Na_2SO_4$ and concentrated to a yellow oil. Purification by flash chromatography (50 mL flash silica gel, 100% chloroform to 15% methanol/ chloroform) gave pure title compound (344 mg, 70% yield). FABMS analysis gave an (MH+) of 677 (compared to the value of 676.97 calculated for the compound $C_{36}H_{56}N_2O_6S_2$). Nuclear magnetic resonance characterization experiments yielded the following molecular signature:
$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 7.20 (d, 4H, J=7), 6.79 (d, 4H, J=7), 3.75 (S, 3H), 3.74 (S, 3H), 3.68 (M, 4H), 3.35 (M, 4H), 2.65 (M, 2H), 2.53 (M, 4H), 2.31 (M, 2H), 1.59 (M, 2H), 1.43 (S, 11H), 1.30 (S, 6H), 1.26 (S, 6H)

**C. Synthesis of BAT-BM (*N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide)**

BAT-BM was prepared as follows. BAT acid ($N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanoicacid)(10.03g, 10.89 mmol) and 75mL of dry methylene chloride ($CH_2Cl_2$) were added to a 250mL round-bottomed flask equipped with magnetic stir bar and argon balloon. To this solution was added diisopropylcarbodiimide (3.40mL, 21.7 mmol, 199 mole%), followed by N-hydroxy-succinimide (3.12g, 27.1 mmol, 249 mole%). This solution was observed to become cloudy within 1h, and

was further incubated with stirring for a total of 4h at room temperature. A solution of *tris*(2-aminoethyl)amine (30mL, 200 mmol, 1840 mole%) in 30mL methylene chloride was then added and stirring continued overnight. The reaction mixture was then concentrated under reduced pressure, and the residue partitioned between ethylacetate (150mL) and 0.5M potassium carbonate ($K_2CO_3$; 150mL). The organic layer was separated, washed with brine and concentrated to give the crude product *N*-[2-(*N'*,*N'*-*bis*(2-aminoethyl)aminoethyl)]-$N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide as a foam/oil.

This crude product was added to a 1000mL round-bottomed flask, equipped with magnetic stir bar, containing 300mL THF, and then 30mL saturated sodium bicarbonate ($NaHCO_3$), 100mL water and N-methoxycarbonylmaleimide (6.13g, 39.5 mmol, 363 mole%) were added. This heterogeneous mixture was stirred overnight at room temperature. THF was removed from the mixture by rotary evaporation, and the aqueous residue was twice extracted with ethylacetate (2X 75mL). The combined organic layers of these extractions were washed with brine, dried over sodium sulfate, filtered through a medium frit and concentrated to about 12g of crude product. Purification by liquid chromatography (250g silicon dioxide/ eluted with a gradient of chloroform → 2% methanol in chloroform) afforded 5.3g of pure product (*N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl)]-$N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide (equivalent to 40% yield), along with approximately 5g of crude product that can be re-purified to yield pure product. Chemical analysis of the purified product confirmed its identity as BAT-BM as follows:

$^1$H NMR (200 mHz, $CDCl_3$): δ 0.91 (12H,s), 1.38 (9H,s), 1.2-1.6 (4H,m), 2.06 (2H,s), 2.18 (2H,t,J=7), 2.31 (4H,m), 2.55 (2H,t,J=5), 2.61 (4H,t,J=6), 2.99 (2H,s), 3.0-3.3 (4H,m), 3.46 (4H,t,J=6), 6.49 (-NH,t,J=4), 6.64 (4H,s), 7.1-7.3 (18H,m), 7.6 (12H,t,J=17).

D. Synthesis of [BAT]-conjugated(εN) Lys(αN-Fmoc) [*N*-ε-($N^9$-*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*[2-methyl-2-(triphenylmethylthio)propyl]-6,9-

diazanonanoyl)-*N*-α-Fmoc-lysine

A 100mL single-necked round-bottomed flask, equipped with stir bar and argon balloon, was charged with $N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$- *bis*[2-methyl-2(triphenylmethylthio)propyl]-6,9-diazanonanoic acid (BAT acid; 3.29g, 3.57 mmol) in 50mL $CH_2Cl_2$ at room temperature. To this was added diisopropylcarbodiimide (DIC; 580μL, 3.70 mmol, 104 mole%) followed immediately by *N*-hydroxysuccinimide (HOSu; 432mg, 3,75 mmol, 105 mole%). The reaction was stirred overnight at room temperature during which time a white precipitate developed. The mixture was filtered and the filtrate concentrated to a solid foam. The crude foam, in a 100mL round-bottomed flask, was dissolved in 75mL of a 2:1 mixture of dimethoxyethane and water. To this homogeneous solution was added *N*-α-Fmoc-lysine hydrochloride (1.52g, 3,75 mmol, 105 mole%) followed by $K_2CO_3$ (517mg, 3,74 mmol, 105 mole%), and the yellow solution stirred overnight at room temperature. The solution was then poured into a 250mL erlenmeyer flask containing 100mL of ethyl acetate and 100mL of water. The organic layer was separated and the aqueous layer further extracted with 50mL ethyl acetate. The combined organic layers were washed once with brine (100mL), dried over $Na_2SO_4$ and concentrated to a yellow solid. This crude product was purified by low-pressure liquid chromatography (150g $SiO_2$, eluted with $CHCl_3$ -> 10% methanol in $CHCl_3$). In this way, 3.12g of the named compound was prepared (69% yield). Chemical analysis of the purified product confirmed its identity as follows: $^1$H NMR (300MHz, $CDCl_3$): δ 0.88 (12H,*s*,broad), 1.05-1.45 (19H,*m*), 1.8-2.1 (4H,*m*), 1.8-2.47 (4H,*m*), 2.75-3.2 (6H,*m*), 3.9-4.3 (4H,*m*,), 7.2 (22H,*m*), 7.6 (16H,*s*,bound). FABMS $MH^+$ was predicted to be 1270.6 and found to be 1272.

**E. Synthesis of BAM ($N^1$-(*t*-butoxycarbonyl)-$N^4$,$N^4$-*bis*[2-methyl-2-(triphenylmethylthio)propyl]-1,4,10-triazadecane**

A 250mL single-necked round-bottomed flask, equipped with a stir bar, reflux condenser and argon balloon, was charged with $N^4$,$N^4$-*bis*[2-methyl-2-

A 250mL single-necked round-bottomed flask, equipped with stir bar and reflux condenser, was charged with 9-phthalimido-$N^4$-(*t*-butoxycarbonyl)-$N^1$,$N^4$-*bis*[2-methyl-2-(triphenylmethylthio)propyl]-1,4-diazanonane (5.50g, 5.319.43 mmol) in 25mL of THF. To this was added 100mL ethanol and 5mL water. The addition of water caused the starting material to precipitate out of solution. Hydrazine hydrate (1.2mL, 24.7 mmol, 466 mole%) was added, and the reaction heated at reflux for two days. The reaction mixture was concentrated and partitioned between 100mL each of water and 0.25M $K_2CO_3$. The organic layer was separated and washed once with brine (75mL), dried over $Na_2SO_4$ and concentrated to a solid foam. Purification of the crude product by low-pressure liquid chromatography (100g $SiO_2$, $CHCl_3$ -> 5% methanol in $CHCl_3$, the column pre-treated with 200mL 2% triethylamine in $CHCl_3$) afforded 3.27g of pure $N^4$-(*t*-butoxycarbonyl)-$N^1$,$N^4$-*bis*[2-methyl-2-(triphenylmethylthio)propyl]-1,4,10-triazadecane as a yellow foam (68% yield). Chemical analysis of the purified product confirmed its identity as follows: $^1$H NMR (300MHz, $CDCl_3$): $\delta$ 0.9 (12H,*s*), 1.2 (6H,*s*), 1.36 (9H,*s*), 2.05 (4H,*m*), 2.24 (2H,*t*), 2.31 (2H,*t*), 2.62 (3H,*t*), 3.0 (2H,*s*,broad), 3.1 (2H,*s*,broad), 7.2 (18H,*m*), 7.6 (12H,*t*). FABMS $MH^+$ was predicted to be 905.5 and found to be 906.5.

## EXAMPLE 2

### Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) was carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with dicyclohexylcarbodiimide/hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/ hydroxybenzotriazole (HBTU/HOBT), and using *p*-hydroxymethylphenoxy-methylpolystyrene (HMP) resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides. Resin-bound products were routinely cleaved using a solution comprised of trifluoroacetic acid, water, thioanisole,

ethanedithiol, and triethylsilane, prepared in ratios of 100 : 5 : 5 : 2.5 : 2 for 1.5 - 3 h at room temperature.

Where appropriate αN-formyl groups were introduced by treating the cleaved, deprotected peptide with excess acetic anhydride in 98% formic acid and stirring for about 18 hours followed by HPLC purification. Where appropriate N-terminal acetyl groups were introduced by treating the free N-terminal amino peptide bound to the resin with 20% v/v acetic anhydride in NMP (N-methylpyrrolidinone) for 30 min.. Where appropriate, 2-chloroacetyl and 2-bromoacetyl groups were introduced either by using the appropriate 2-halo-acetic acid as the last residue to be coupled during SPPS or by treating the N-terminus free amino peptide bound to the resin with either the 2-halo-acetic acid/ diisopropylcarbodiimide/ N-hydroxysuccinimide in NMP of the 2-halo-acetic anhydride/ diisopropylethylamine in NMP. Where appropriate, HPLC-purified 2-haloacetylated peptides were cyclized by stirring an 0.1 - 1.0 mg/mL solution in bicarbonate or ammonia buffer (pH 8) with or without 0.5 - 1.0 mM EDTA for 1 - 48 hours, followed by acidification with acetic acid, lyophilization and HPLC purification. Where appropriate, Cys-Cys disulfide bond cyclizations were performed by treating the precursor cysteine-free thiol peptides at 0.1mg/mL in pH 7 buffer with aliquots of 0.006 M $K_3Fe(CN)_6$ until a stable yellow color persisted. The excess oxidant was reduced with excess cysteine, the mixture was lyophilized and then purified by HPLC.

Where appropriate the "Pica" group was introduced by conjugating picolylamine to a precursor peptide using diisopropylcarbodiimide and N-hydroxysuccinimide. Where appropriate BAT ligands were introduced either by using the appropriate BAT acid as the last residue to be coupled during SPPS or by treating the N-terminus free amino peptide bound to the resin with BAT acid/ diisopropylcarbodiimide/ N-hydroxysuccinimide in NMP. Where appropriate, [BAM] was conjugated to the peptide by first activating the peptide carboxylate with a mixture of diisopropylcarbodiimide/N-hydroxysuccinimide or HBTU/HOBt in DMF, NMP or $CH_2Cl_2$, followed by coupling in the presence of diisopropylethylamine; after coupling, the conjugate was deprotected as

described above.

Where appropriate, BSME adducts were prepared by reacting single thiol-containing peptides (5 to 50 mg/mL in 50 mM sodium phosphate buffer, pH 8) with 0.5 molar equivalents of BMME (*bis*-maleimidomethylether) pre-dissolved in acetonitrile at room temperature for approximately 1-18 hours. The solution was concentrated and the product was purified by HPLC.

Where appropriate, TSEA adducts were prepared by reacting single thiol-containing peptide (at concentrations of 10 to 100 mg/mL peptide in DMF, or 5 to 50 mg/mL peptide in 50mM sodium phosphate (pH 8)/ acetonitrile or THF) with 0.33 molar equivalents of TMEA (*tris*(2-maleimidoethyl)amine; Example 2) pre-dissolved in acetonitrile or DMF, with or without 1 molar equivalent of triethanolamine, at room temperature for approximately 1-18h. Such reaction mixtures containing adducts were concentrated and the adducts were then purified using HPLC.

Where appropriate, BAT-BS adducts were prepared by reacting single thiol-containing peptide (at concentrations of 2 to 50 mg/mL peptide in 50mM sodium phosphate (pH 8)/ acetonitrile or THF) with 0.5 molar equivalents of BAT-BM (*N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl)]-$N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$,-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide;Example 1) pre-dissolved in acetonitrile or THF, at room temperature for approximately 1-18h. The solution was then evaporated to dryness and [BAT-BS]-peptide conjugates deprotected by treatment with 10mL TFA and 0.2mL triethylsilane for 1h. The solution was concentrated, the product adducts precipitated with ether, and then purified by HPLC.

Crude peptides were purified by preparative high pressure liquid chromatography (HPLC) using a Waters Delta Pak C18 column and gradient elution using 0.1% trifluoroacetic acid (TFA) in water modified with acetonitrile. Acetonitrile was evaporated from the eluted fractions which were then lyophilized. The identity of each product was confirmed by fast atom bombardment mass spectroscopy (FABMS).

## EXAMPLE 3

### A General Method for Radiolabeling with Tc-99m

0.1 mg of a peptide prepared as in Example 2 was dissolved in 0.1 mL of water or 50 mM potassium phosphate buffer (pH = 5, 6 or 7.4). Tc-99m gluceptate was prepared by reconstituting a Glucoscan vial (E.I. DuPont de Nemours, Inc.) with 1.0 mL of Tc-99m sodium pertechnetate containing up to 200 mCi and allowed to stand for 15 minutes at room temperature. 25 $\mu$l of Tc-99m gluceptate was then added to the peptide and the reaction allowed to proceed at room temperature or at 100°C for 15-30 min and then filtered through a 0.2 $\mu$m filter.

The Tc-99m labeled peptide purity was determined by HPLC using the following conditions: a Waters DeltaPure RP-18, 5$\mu$, 150mm x 3.9mm analytical column was loaded with each radiolabeled peptide and the peptides eluted at a solvent flow rate equal to 1 mL/min. Gradient elution was performed beginning with 10% solvent A (0.1% CF3COOH/$H_2O$) to 40% solvent $B_{90}$ (0.1% $CF_3COOH$/90% $CH_3CN$/$H_2O$) over the course of 20 min.

Radioactive components were detected by an in-line radiometric detector linked to an integrating recorder. Tc-99m gluceptate and Tc-99m sodium pertechnetate elute between 1 and 4 minutes under these conditions, whereas the Tc-99m labeled peptide eluted after a much greater amount of time.

The following Table illustrates successful Tc-99m labeling of peptides prepared according to Example 2 using the method described herein.

- 29 -

| Peptides | FABMS MH⁺ | Radiochemical Yield(%)* | HPLC R(min)** |
|---|---|---|---|
| formyl-MLFC$_{Acm}$G.Pica | 760 | 100 | 10.9, 11.5, 12.2 |
| Pic.GC$_{Acm}$(VGVAPG)$_3$amide | 1795 | 100 | 12.4 |
| Pic.GC$_{Acm}$(VPGVG)$_4$amide | 1992 | 100 | 12.0 |
| Pic.GC$_{Acm}$RALVDTLKFVTQAEGAKamide | 2183 | 95 | 17.2 |
| Pic.GC$_{Acm}$RALVDTEFKVKQEAGAKamide | 2226 | 96 | 15.5 |
| Pic.GC$_{Acm}$PLARITLPDFRLPEIAIPamide | 236 | 92 | 19.2 |
| Pic.GC$_{Acm}$GQQHHLGGAKAGDV | 1838 | 48 | 12.8-16.6 |
| Pic.GC$_{Acm}$PLYKKIIKKLLES | 1910 | 81 | 10.7-14.5 |
| Pic.GC$_{Acm}$LRALVDTLKamide | 1363 | 92 | 13.0-14.5 |
| Pic.GC$_{Acm}$GGGLRALVDTLKamide | 1535 | 100 | 15.6 |
| Pic.GC$_{Acm}$GGGLRALVDTLKFVTQAEGAKamide | 2354 | 92 | 15.1 |
| Pic.GC$_{Acm}$GGGRALVDTLKALVDTLamide | 2035 | 86 | 14.5 |
| Pic.GC$_{Acm}$GHRPLDKKREEAPSLRPAPPPISGGGYR | 3377 | 94 | 11.3 |
| Pic.GC$_{Acm}$PSPSPIHPAHHKRDRRQamide | 2351 | 94 | 11.2, 14.4 |
| Pic.GC$_{Acm}$GGGF$_D$.Cpa.YW$_D$KTFTamide | 1681 | 98 | 13.8-16.8 |
| Pic.GC$_{Acm}$GGCNP.Apc.GDC | 1217 | 69 | 6.6-13.7 |
| (Pic.SC$_{Acm}$SYNRGDSTC)$_3$-TSEA | 4488 | 99 | 10.4, 11.2 |
| Pic.GC$_{Mob}$GGGLRALVDTLKamide | 1471 | 100 | 11.9 |
| Pic.GCGGGLRALVDTLKamide | 1350 | 100 | 11.2, 11.6 |

| Peptides | FABMS MH+ | Radiochemical Yield(%)* | HPLC $R_t$(min)** |
|---|---|---|---|
| Pic.GCYRALVDTLKFVTQAEGAKamide | 2275 | 95 | 18.6, 19.1 |
| Pic.GC(VGVAPG)$_3$amide | 1724 | 95 | 17.3 |
| [BAT]GGPLYKKIIKKLLES | 2006 | 94 | 9.5 |
| [BAT].Hly.GDP.Hly.GDF.amide | 1209 | 99 | 10.8 |
| [BAT]GHRPLDKKREEAPSLRPAPPPISGGGYR.amide | 3357 | 93 | 10.4, 11.6 |
| [BAT]PKLEELKEKLKELLEKLKEKLA | 2969 | 90 | 12.3 |
| [BAT]G.Apc.GDV.Apc.GDFK.amide | 1432 | 97 | 17.5 |
| [BAT]PLARITLPDFRLPEIAIP.amide | 2350 | N.D | N.D |
| [BAT]RALVDTEFKVKQEAGAK.amide | 2208 | 96 | 12.1 |
| [BAT]YRALVDTLKFVTQAEGAK.amide | 2329 | 96 | 13.3 |
| [BAT]VPGVGVPGVGVPGVGVPGVG.amide | 1974 | 96 | 11.9, 12.8 |
| [BAT]RALVDTLKFVTQAEGAKamide | 2165 | 98 | 19.0 |
| *formyl*-MLFK[BAT].amide | 884 | 99 | 12.6 |
| *formyl*.Thp.LF[BAM] | 775 | 99 | 13.3, 13.6 |
| ($CH_3$-N)-FYW$_D$KVE[BAM] | 1171 | 98 | 12.3, 13.6 |
| *formyl*-MLFK[BAT] | 884 | 96 | 11.9, 12.8 |
| *formyl*-MLFK[BAT]KKKKK.amide | 1524 | 96 | 11.7, 12.2 |
| *formyl*-MLFK[BAT]GSGSGS.amide | 1315 | 97 | 11.9, 12.8 |
| *formyl*-MLFK[BAT]EGE | 1013 | 99 | 12.3 |

| Peptides | FABMS MH⁺ | Radiochemical Yield(%)* | HPLC $R_t$(min)** |
|---|---|---|---|
| *formyl*-M.Dpg.F[BAM] | 770 | 98 | 13.7 |
| *formyl*-MLFK[BAT]E | 1200 | 98 | 13.7 |
| (*formyl*-MLFK[BAT]GGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-BSME | 3477 | 99 | 11.9, 12.4 |
| [BAT]RALVDTLKKLKKKL.amide | 2135 | 97 | 11.9 |
| (CH$_2$CO-Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGC.amide)$_2$-[BAT-BS] | 3409 | 98 | 10.3 |
| [BAT](VGVAPG)$_3$.amide | 1778 | 98 | 10.3 |
| YRALVDTLKFVTQAEGAK[BAT].amide | 2329 | 98 | 11.4 |
| K[BAT]D.Nal.C$_{Me}$YW$_D$KVC$_{Me}$T.amide | 1573 | 97 | 12.0, 12.5 |
| [DTPA](D-Nal.SYW$_D$KVTK[BAT])$_2$.amide | 3210 | 97 | 12.1, 12.5 |
| [DTPA](D-Nal.SYW$_D$KVTK[BAT]).amide | 1801 | 96 | 11.8, 12.0 |
| [DTPA]K[BAT]D-Nal.C$_{Me}$YW$_D$KVC$_{Me}$T.amide | 1949 | 96 | 11.8, 12.0 |
| [BAT-BS](ma)GGGRALVDTLKFVTQAEGAK.amide | 4808 | 96 | 12.0 |
| [BAT]KKLLKKLYKKIIKKLLES | 2533 | 99 | bound |
| *p*Glu.GVNDNEEGFFSARK[BAT].amide | 1997 | N.D | N.D. |

* The following labeling conditions were used with the appropriate peptides:

1. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 7.4) and labeled at room temperature.
2. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 7.4) and labeled at 100°C.
3. The peptide is dissolved in water and labeled at room temperature.
4. The peptide is dissolved in water and labeled at 100°C.
5. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 6.0) and labeled at 100°C.
6. The peptide is dissolved in 50 mM potassium phosphate buffer (pH 5.0) and labeled at room temperature.

** HPLC methods:

general: solvent A = 0.1% $CF3COOH/H_2O$
solvent $B_{70}$ = 0.1% $CF_3COOH$/70% $CH_3CN/H_2O$
solvent $B_{90}$ = 0.1% $CF_3COOH$/90% $CH_3CN/H_2O$
solvent flow rate = 1 mL/min

Vydak column = Vydak 218TP54 RP-18, 5$\mu$ x 220mm x 4.6mm analytical column with guard column
Brownlee column = Brownlee Spheri-5 RP-18, 5$\mu$ x 220mm x 4.6mm column
Waters column = Waters Delta-Pak C18, 5$\mu$m, 39 X 150mm

| | | |
|---|---|---|
| Method 1: | Brownlee column | 100% A to 100% $B_{70}$ in 10 min |
| Method 2: | Vydak column | 100% A to 100% $B_{90}$ in 10 min |
| Method 3: | Vydak column | 100% A to 100% $B_{70}$ in 10 min |
| Method 4: | Brownlee column | 100% A to 100% $B_{90}$ in 10 min |
| Method 5: | Waters column | 100% A to 100% $B_{90}$ in 10 min |

Single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry* (2d. ed.), 1988 (MacMillen Publishing: New York) p.33; Ac = acetyl; Pic = picolinoyl (pyridine-2-carbonyl) = 6-aminocaproic acid; Hly = homolysine; Acm = acetamidomethyl; *p*Glu = *pyro*-glutamic acid; Mob = 4-methoxybenzyl; Pica = picolylamine (2-(aminomethyl)pyridine); Apc = L-[S-(3-aminopropyl)cysteine; $F_D$ = D-phenylalanine; $W_D$ = D-tryptophan; $Y_D$ = D-tyrosine; Cpa = L-(4-chlorophenyl)alanine; Thp = 4-amino-tetrahydrothiopyran-4-carboxylic acid; ma = mercaptoacetic acid; D-Nal = D-2-naphthylalanine; Dpg = dipropylglycine; Nle = norleucine;
BAT = $N^6$,$N^9$-*bis*(2-mercapto-2-methylpropyl)-6,9-diazanonanoic acid; BAT acid (protected) = $N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanoic acid; BAM = $N^1$,$N^4$-*bis*(2-mercapto-2-methylpropyl)-1,4,10-triazadecane; BAM (protected) = $N^1$-(*t*-butoxycarbonyl)-$N^1$,$N^4$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-1,4,10-

triazadecane; [BAT-BM] = *N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl]-$N^9$-(*t*-butoxycarbonyl)-$N^6$,$N^9$-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide; [BAT-BS] = *N*-[2-(*N'*,*N'*-*bis*(2-succinimidoethyl)aminoethyl]-$N^6$,$N^9$-*bis*(2-mercapto-2-methylpropyl)-6,9-diazanonanamide; [BMME] = *bis*-maleimidomethylether; [BSME] = *bis*-succinimidomethylether; [DTPA] = diethylenetriaminepentaacetic acid

EXAMPLE 4

Localization and *In Vivo* Imaging of Atherosclerotic Plaque using Tc-99m Labeled Compound P215 in the Hypercholesterol Rabbit Model

Twenty-two New Zealand White (NZW) rabbits of both sexes and weighing 2-3kg were divided into two groups. The control group consisted of 6 rabbits that were housed and fed commercial rabbit chow (Purina). Sixteen rabbits, the HC group, were fed a standardized, cholesterol-rich diet (rabbit chow mixed to a 1% w/w concentration of cholesterol) from seven weeks until 28 weeks of age. All animals were given water *ad libitum*.

Tc-99m labeled P215 ([BAT]RALVDTLKFVTQAEGAK.amide) was prepared as described above. Approximately 250-400μg of peptide was labeled with 140-160mCi of Tc-99m and prepared in unit doses of 7-8mCi (12.5-20.0 μg/rabbit; 6-7μg/kg) in 0.2mL volume doses. Adult rabbits were dosed with Tc-99m labeled peptide intravenously in a lateral ear vein by slow bolus infusion (approximately 0.1mL/min). A gamma camera fitted with a pin-hole collimator (5mm aperture) and energy window set for Tc-99m and programmed to accumulate 500,000 counts or scan for a desired time. Shortly before imaging, animals were anesthetized with a mixture of ketamine and xylazine (5:1, 1mL/kg intramuscularly).

Gamma camera images were collected at 40°-45° just above the heart (left anterior oblique [LAO] view) to delineate the aortic arch and view the descending aorta. Images were acquired at 1 and 2h and occasionally at 3 and 5h after injection. Supplementary anesthesia was injected as needed prior to each image collection.

At 2.5 h (after a 2h scan), animals were sacrificed with an intravenous dose of sodium pentobarbital. Upon necropsy, the aorta was removed and branching vessels dissected free from the aortic valve to the mid-abdominal region. Using a parallel hole collimator, the aorta was imaged *ex corpora*. Next, the aortae were opened longitudinally and stained with Sudan IV, thereby turning atherosclerotic plaque a deep red brick color. Lipid-free and uninjured aortic endothelium retains its normal, glistening white-pink appearance under these conditions.

The results of these experiments are shown in Figures 1-3. Both groups of rabbits showed rapid systemic clearance of Tc-99m P215. The scintigraphic images indicate that the hepatobilliary system provides the principal clearance pathway. Control (plaque-free) aortae were only visible for a short time after injection, resulting from circulating, blood-borne radioactivity. Each of the HC-fed NZW rabbit aortae showed a unique pattern and intensity of plaque distribution when imaged *ex corpora*. All the HC aortae had variable amounts of radioactivity accumulation but were consistent in their display of the greatest deposition in the region of the aortic arch, with lesser degrees of accumulation in the distal and proximal segments of the aorta.

Positive correlations were observed among the *in vivo* and *ex corpora* Tc-99m P215 images and the deposition patterns of Sudan IV in the HC-treated rabbit aortae. In contrast, no control aortae showed any regional uptake of labeled peptide. Figure 1 shows the deposition pattern of Sudan IV in 1 HC-treated and 4 control rabbit aortae. The dark areas indicate the location of atherosclerotic plaque. Figure 2 and 3 show the corresponding *in vivo* and *ex corpora* images, respectively.

These results demonstrate that Tc-99m labeled P215 is capable of imaging atherosclerotic plaque in an animal with high uptake and rapid clearance, facilitating early observation. Additionally, normal aortic tissue shows minimal uptake of labeled P215, thereby reducing the likelihood of artifactual positive scintigraphic images.

## EXAMPLE 5

### *In Vivo* Imaging using Tc-99m Labeled Compound P357 of Deep Vein Thrombosis in a Canine Model

Mongrel dogs (25-35lb., fasted overnight) were sedated with a combination of ketamine and aceprozamine intramuscularly and then anesthetized with sodium pentobarbital intravenously. An 18-gauge angiocath was inserted in the distal half of the right femoral vein and an 8mm Dacron®-entwined stainless steel embolization coil (Cook Co., Bloomington IN) was placed in the femoral vein at approximately mid-femur in each animal. The

catheter was removed, the wound sutured and the placement of the coil documented by X-ray. The animals were then allowed to recover overnight.

One day following coil placement, each animal was re-anesthetized, intravenous saline drips placed in each foreleg and a urinary bladder catheter inserted to collect urine. The animal was placed supine under a gamma camera which was equipped with a low-energy, all purpose collimator and photopeaked for Tc-99m. Images were acquired on a NucLear Mac computer system.

Tc-99m labeled P357 [$(CH_2CO\text{-}Y_D.Apc.GDCGGC_{Acm}GC_{Acm}GGC.amide)_2$-[BAT-BS]] [185-370 mBq (5-10 mCi) Tc-99m and 0.2-0.4mg P357] was injected into one foreleg intravenous line at its point of insertion. The second line was maintained for blood collection. Anterior images over the legs were acquired for 500,000 counts or 20 min (whichever was shorter), at approximately 10-20 min, and at approximately 1, 2, 3 and 4h post-injection. Following the collection of the final image, each animal was deeply anesthetized with pentobarbital. Two blood samples were collected on a cardiac puncture using a heparinized syringe followed by a euthanasing dose of saturated potassium chloride solution administered by intercardiac or bolus intravenous injection. The femoral vein containing the thrombus and samples of thigh muscle were then carefully dissected out. The thrombus was then dissected free of the vessel and placed in a pre-weighed test tube. The thrombus samples were then weighed and counted in a gamma well counter in the Tc-99m channel. Known fractions of the injected doses were counted as well.

Fresh thrombus weight, percent injected dose (%ID)/g in the thrombus and blood obtained just prior to euthanasia and thrombus/blood and thrombus/muscle ratios determined. Thrombus/background ratios were determined by analysis of the counts/pixel measured in regions-of-interest (ROI) drawn over the thrombus and adjacent muscle from computer-stored images.

Deep vein thrombus imaging was studied in a total of eight dogs. Tissue data from these experiments are shown in the following Table.

Representative examples of images acquired in an anterior view over the hind legs of one dog at 23, 71, 139, 208 and 222 min is presented in Figure 4. These images show indications of labeled peptide uptake as early as 23 min post-injection, with unequivocal localization by 71 min which persisted until the end of the imaging period.

These results demonstrate that deep vein thrombi can be rapidly and efficiently located *in vivo*. Localization was clearly established within 1h post-injection and persisted, with increasing contrast and focal definition, over nearly 4h post-injection.

| $IC_{50}$ | Thrombus/ Background | %ID/g Thrombus | Thrombus/Blood | Thrombus/Muscle |
|---|---|---|---|---|
| 0.081* | 2.3* | 0.016 ± 0.005 | 5.6 ± 1.4 | 17 ± 4.7 |

Values shown are the average ± the standard deviation from the mean; [*=n=1]

The $IC_{50}$ value shown in the Table was determined as follows. Platelet aggregation studies were performed essentially as described by Zucker (1989, Methods in Enzymol. 169: 117-133). Briefly, platelet aggregation was assayed with or without putative platelet aggregation inhibitory compounds using fresh human platelet-rich plasma, comprising 300,000 platelets per microlitre. Platelet aggregation was induced by the addition of a solution of adenosine diphosphate to a final concentration of 10 to 15 micromolar, and the extent of platelet aggregation monitored using a Bio/Data aggregometer (Bio/Data Corp., Horsham, PA). The concentrations of platelet aggregation inhibitory compounds used were varied from 0.1 to 500 $\mu$g/mL. The concentration of inhibitor that reduced the extent of platelet aggregation by 50% (defined as the $IC_{50}$) was determined from plots of inhibitor concentration *versus* extent of platelet aggregation. An inhibition curve for peptide RGDS was determined for each batch of platelets tested as a positive control.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth

in the appended claims.

What is claimed is:

1. A reagent for preparing a scintigraphic imaging agent for imaging sites within a mammalian body, comprising a specific binding peptide having an amino acid sequence comprising 3 to 100 amino acids and a radiolabel-binding moiety covalently linked thereto, wherein the radiolabel-binding moiety is capable of forming a complex with a radioisotope, wherein the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral.

2. The reagent of Claim 1 wherein the specific binding peptide and the radiolabel-binding moiety are covalently linked through from about one to about 20 amino acids.

3. The reagent of Claim 1 wherein the radioisotope is technetium-99m.

4. The reagent of Claim 1 wherein the specific binding peptide is selected from the group consisting of peptides having the amino acid sequences:

formyl-MLF
(VGVAPG)$_3$amide
(VPGVG)$_4$amide
RALVDTLKFVTQAEGAKamide
RALVDTEFKVKQEAGAKamide
PLARITLPDFRLPEIAIPamide
GQQHHLGGAKAGDV
PLYKKIIKKLLES
LRALVDTLKamide
GGGLRALVDTLKamide
GGGLRALVDTLKFVTQAEGAKamide
GGGRALVDTLKALVDTLamide
GHRPLDKKREEAPSLRPAPPPISGGGYR
PSPSPIHPAHHKRDRRQamide
GGGF$_D$.Cpa.YW$_D$KTFTamide
GGCNP.Apc.GDC
S—S
[SYNRGDSTC]$_3$-TSEA
GGGLRALVDTLKamide
GCGGGLRALVDTLKamide
GCYRALVDTLKFVTQAEGAKamide
GC(VGVAPG)$_3$amide

5. The reagent of Claim 1 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding

compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

6. The reagent of Claim 5 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N'*,*N'*-*bis*(2-succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine or a derivative thereof.

7. A scintigraphic imaging agent comprising the reagent according to Claim 1 wherein the radiolabel binding moiety is bound to a radiolabel.

8. The reagent of Claim 6 wherein the radiolabel is technetium-99m.

9. A complex formed by reacting the reagent of Claim 1 with technetium-99m in the presence of a reducing agent.

10. The complex of Claim 9, wherein the reducing agent is selected from the group of a dithionite ion, a stannous ion, or a ferrous ion.

11. A complex formed by labeling the reagent of Claim 1 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

12. A kit for preparing a radiopharmaceutical preparation, said kit comprising sealed vial containing a predetermined quantity of the reagent of Claim 1 and a sufficient amount of reducing agent to label the reagent with technetium-99m.

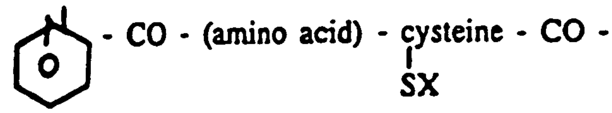
13. A method for imaging a site of within a mammalian body comprising administering an effective diagnostic amount of the reagent of Claim 1 that is labeled with technetium-99m and detecting the Tc-99m localized at the site within the mammalian body.

14. The process of preparing the reagent according to Claim 1 wherein the peptide is chemically synthesized *in vitro*.

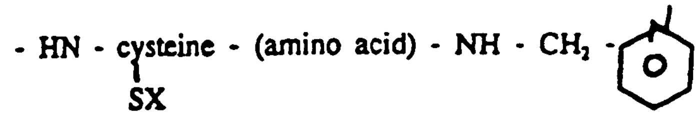
15. The process of preparing the peptide according to Claim 14 wherein the peptide is synthesized by solid phase peptide synthesis.

16. A reagent for preparing a scintigraphic imaging agent for imaging sites within a mammalian body, comprising a specific binding peptide having

an amino acid sequence comprising from 3 to 100 amino acids and a radiolabel-binding moiety of formula:




or




wherein X = H or a protecting group;

(amino acid) = any amino acid;

the radiolabel-binding moiety is covalently linked to the peptide and wherein the radiolabel-binding moiety forms a complex with a radioisotope and the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral.

17. The reagent of Claim 16 wherein the amino acid is glycine and wherein X is an acetamidomethyl protecting group.

18. The reagent of Claim 16 wherein the specific binding peptide and the radiolabel-binding moiety are covalently linked through from about one to about 20 amino acids.

19. The reagent of Claim 16 wherein the radioisotope is technetium-99m.

20. The reagent of Claim 16 wherein the specific binding peptide is selected from the group consisting of peptides having the amino acid sequences:

formyl-MLF
$(VGVAPG)_3$amide
$(VPGVG)_4$amide
RALVDTLKFVTQAEGAKamide
RALVDTEFKVKQEAGAKamide
PLARITLPDFRLPEIAIPamide
GQQHHLGGAKAGDV
PLYKKIIKKLLES
LRALVDTLKamide
GGGLRALVDTLKamide
GGGLRALVDTLKFVTQAEGAKamide
GGGRALVDTLKALVDTLamide

GHRPLDKKREEAPSLRPAPPPISGGGYR
PSPSPIHPAHHKRDRRQamide
GGGF$_D$.Cpa.YW$_D$KTFTamide
GGCNP.Apc.GDC
S—S
[SYNRGDSTC]$_3$-TSEA
GGGLRALVDTLKamide
GCGGGLRALVDTLKamide
GCYRALVDTLKFVTQAEGAKamide
GC(VGVAPG)$_3$amide

21. The reagent of Claim 16 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

22. The reagent of Claim 21 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N'*,*N'*-*bis*(2-succinimido-ethyl)aminoethyl)]-*N*$^6$,*N*$^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine or a derivative thereof.

23. A scintigraphic imaging agent comprising the reagent according to Claim 16 wherein the radiolabel binding moiety is bound to a radiolabel.

24. The reagent of Claim 23 wherein the radiolabel is technetium-99m.

25. A complex formed by reacting the reagent of Claim 16 with technetium-99m in the presence of a reducing agent.

26. The complex of Claim 25, wherein the reducing agent is selected from the group of a dithionite ion, a stannous ion, or a ferrous ion.

27. A complex formed by labeling the reagent of Claim 16 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

28. A kit for preparing a radiopharmaceutical preparation, said kit comprising sealed vial containing a predetermined quantity of the reagent of Claim 16 and a sufficient amount of reducing agent to label the reagent with technetium-99m.

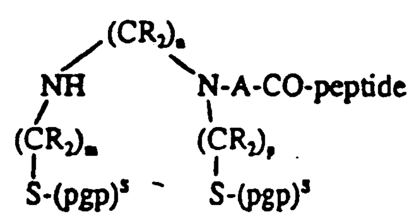
29. A method for imaging a site of within a mammalian body comprising administering an effective diagnostic amount of the reagent of Claim 16 that is labeled with technetium-99m and detecting the Tc-99m localized at the site within the mammalian body.

30. The process of preparing the reagent according to Claim 16 wherein the peptide is chemically synthesized *in vitro*.

31. The process of preparing the peptide according to Claim 30 wherein the peptide is synthesized by solid phase peptide synthesis.

32. A reagent for preparing an scintigraphic imaging agent for imaging sites within a mammalian body, comprising a specific binding peptide having an amino acid sequence comprising from 3 to 100 amino acids, and a bisamino bisthiol radiolabel-binding moiety covalently linked thereto, wherein the radiolabel-binding moiety forms a complex with a radioisotope, the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral, and wherein the bisamino bisthiol radiolabel-binding moiety has a formula selected from the group consisting of:

I.



wherein each R is independently H, $CH_3$ or $C_2H_5$;

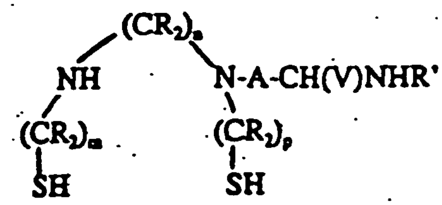
each $(pgp)^s$ is independently a thiol protecting group or H;

m, n and p are independently 2 or 3;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

and

II.



wherein each R is independently H, $CH_3$ or $C_2H_5$;

m, n and p are independently 2 or 3;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

V = H or -CO-peptide;

R' = H or peptide;

and wherein when V = H, R' = peptide and when R' = H, V = -CO-peptide.

33. The peptide of Claim 32 wherein the specific binding peptide and the radiolabel-binding moiety are covalently linked through from about one to about 20 amino acids.

34. The peptide of Claim 32 wherein the radioisotope is technetium-99m.

35. The peptide of Claim 32 wherein the specific binding peptide is selected from the group consisting of peptides having the amino acid sequences:

formyl-MLF
(VGVAPG)$_3$amide
(VPGVG)$_4$amide
RALVDTLKFVTQAEGAKamide
RALVDTEFKVKQEAGAKamide
PLARITLPDFRLPEIAIPamide
GQQHHLGGAKAGDV
PLYKKIIKKLLES
LRALVDTLKamide
GGGLRALVDTLKamide
GGGLRALVDTLKFVTQAEGAKamide
GGGRALVDTLKALVDTLamide
GHRPLDKKREEAPSLRPAPPPISGGGYR
PSPSPIHPAHHKRDRRQamide
GGGF$_D$.Cpa.YW$_D$KTFTamide

GGCNP.Apc.GDC
S—S
[SYNRGDSTC]$_3$-TSEA
GGGLRALVDTLKamide
GCGGGLRALVDTLKamide
GCYRALVDTLKFVTQAEGAKamide
GC(VGVAPG)$_3$amide

36. The reagent of Claim 32 wherein the reagent further comprises a polyvalent linking moiety covalently linked to a multiplicity of specific binding compounds and also covalently linked to a multiplicity of radiolabel-binding moieties to comprise a reagent for preparing a multimeric polyvalent scintigraphic imaging agent, wherein the molecular weight of the multimeric polyvalent scintigraphic imaging agent is less than about 20,000 daltons.

37. The reagent of Claim 36 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, 4-(2,2-dimethylacetyl)benzoic acid, *N*-[2-(*N'*,*N'*-*bis*(2-succinimido-ethyl)aminoethyl)]-$N^6$,$N^9$-*bis*(2-methyl-2-mercaptopropyl)-6,9-diazanonanamide, *tris*(succinimidylethyl)amine or a derivative thereof.

38. A scintigraphic imaging agent comprising the reagent according to Claim 32 wherein the radiolabel binding moiety is bound to a radiolabel.

39. The reagent of Claim 38 wherein the radiolabel is technetium-99m.

40. A complex formed by reacting the reagent of Claim 32 with technetium-99m in the presence of a reducing agent.

41. The complex of Claim 40, wherein the reducing agent is selected from the group of a dithionite ion, a stannous ion, or a ferrous ion.

42. A complex formed by labeling the reagent of Claim 32 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

43. A kit for preparing a radiopharmaceutical preparation, said kit comprising sealed vial containing a predetermined quantity of the reagent of Claim 32 and a sufficient amount of reducing agent to label the reagent with technetium-99m.

44. A method for imaging a site of within a mammalian body comprising administering an effective diagnostic amount of the reagent of Claim

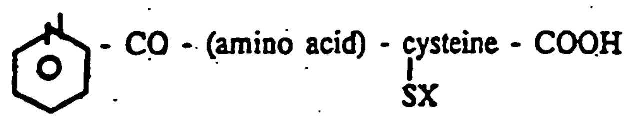
32 that is labeled with technetium-99m and detecting the Tc-99m localized at the site within the mammalian body.

45. The process of preparing the reagent according to Claim 32 wherein the peptide is chemically synthesized *in vitro*.

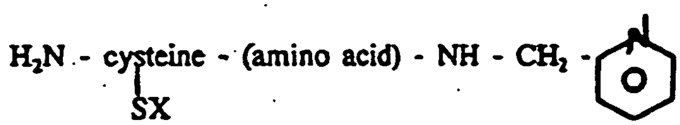
46. The process of preparing the peptide according to Claim 45 wherein the peptide is synthesized by solid phase peptide synthesis.

47. A composition of matter comprising a radiolabel-binding moiety wherein the radiolabel-binding moiety forms a complex with a radioisotope, and wherein the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral.

48. A composition of matter comprising a radiolabel-binding moiety of formula:




or




wherein X = H or a protecting group;

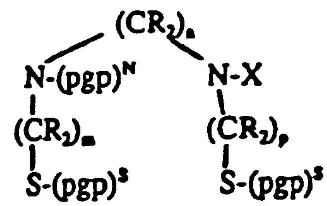
(amino acid) = any amino acid; and

wherein the radiolabel-binding moiety forms a complex with a radioisotope, and the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral.

49. The reagent of Claim 12 wherein the amino acid is glycine and wherein X is an acetamidomethyl protecting group.

50. A composition of matter comprising a bisamino bisthiol radiolabel-binding moiety having a formula selected from the group consisting of:

I.



wherein each R is independently H, $CH_3$ or $C_2H_5$ but if X = H, one R = Y;

$(pgp)^N$ = an amine protecting group or H;

each $(pgp)^S$ is independently a thiol protecting group or H;

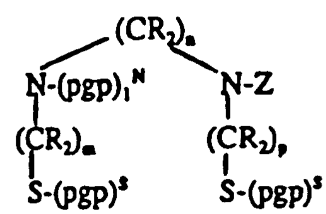
m, n and p are independently 2 or 3;

X = H or -A-COOH, but if X=H, one R = Y and $(pgp)^N$ is not H;

Y = -A-COOH;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

II.



wherein each R is independently H, $CH_3$ or $C_2H_5$ but if Z = H, one R = Y;

each $(pgp)^N$ is an amine protecting group or H;

each $(pgp)^S$ is independently a thiol protecting group or H;

m, n and p are independently 2 or 3;

Z = H or -A-CH(V)NH$(pgp)_2^N$, but if Z = H, one R = Y;

Y = -A-CH(V)NH$(pgp)_2^N$;

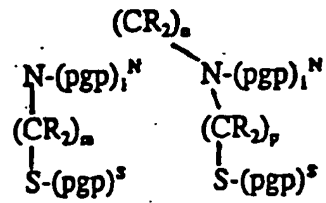
A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

V = H or COOH;

and wherein if $(pgp)^N$ and V are H, then $(pgp)^S$ is not H and if $(pgp)^S$ and V are H, then $(pgp)^N$ is not H, and if V is H, $(pgp)_1^N$ is not H;

and

III.



wherein each R is independently H, $CH_3$ or $C_2H_5$ and one R = Y;

each $(pgp)^N$ is an amine protecting group or H;

each $(pgp)^S$ is independently a thiol protecting group or H;

m, n and p are independently 2 or 3;

Y = -A-CH(V)NH$(pgp)_2^N$;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, combinations or substituted derivatives thereof;

V = H or COOH;

and wherein if $(pgp)_2^N$ and V are H, then $(pgp)^S$ is not H and if $(pgp)^S$ and V are H, then $(pgp)_2^N$ is not H, and at least one $(pgp)_1^N$ moiety is not H; wherein the radiolabel-binding moiety forms a complex with a radioisotope, and the complex of the radiolabel-binding moiety and the radioisotope is electrically neutral.

51. A composition of matter comprising a compound of Claim 50 attached to the ε-amino group of N-α-protected lysine.

52. The composition of matter of Claim 51 which is [*N*-ε-(*N*⁹-*t*-butoxycarbonyl)-*N*⁶,*N*⁹-*bis*[2-methyl-2-(triphenylmethylthio)propyl]-6,9-diazanonanoyl)-*N*-α-Fmoc-lysine.

Fig. 1

Fig. 2

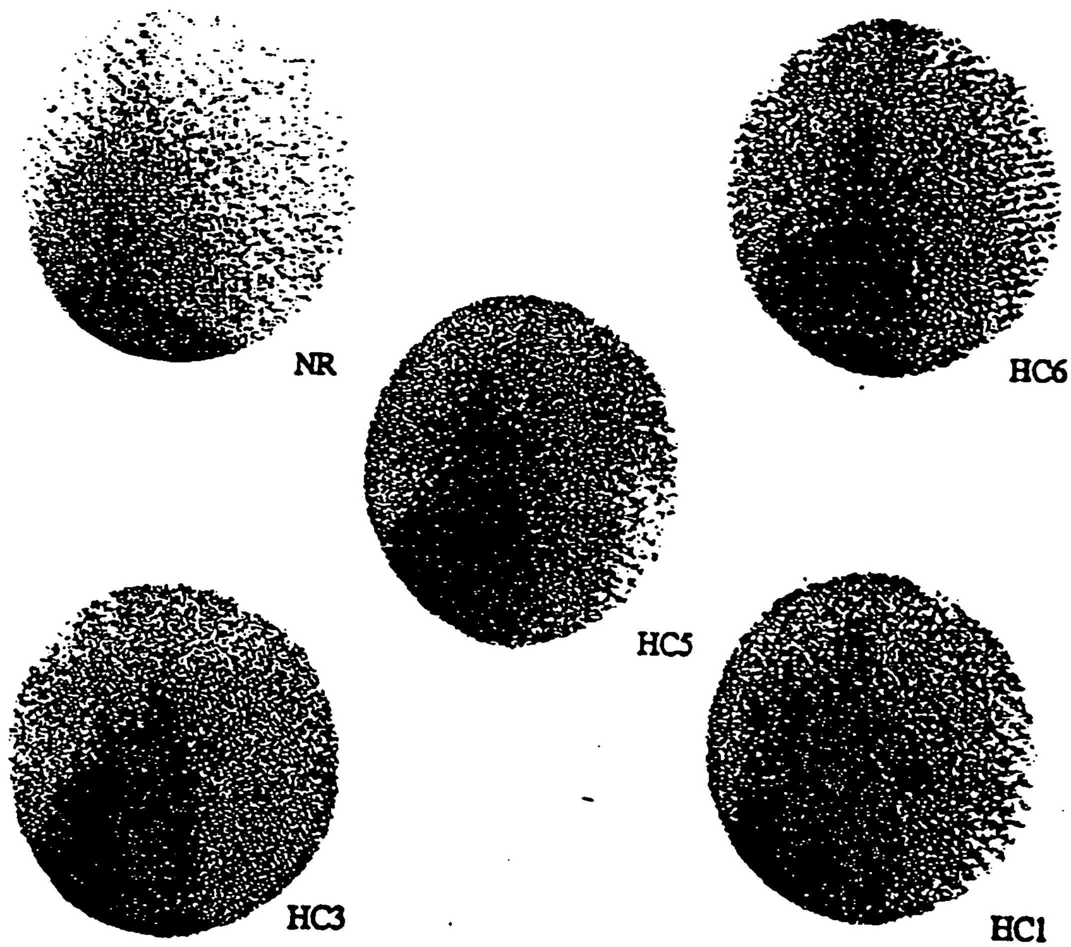
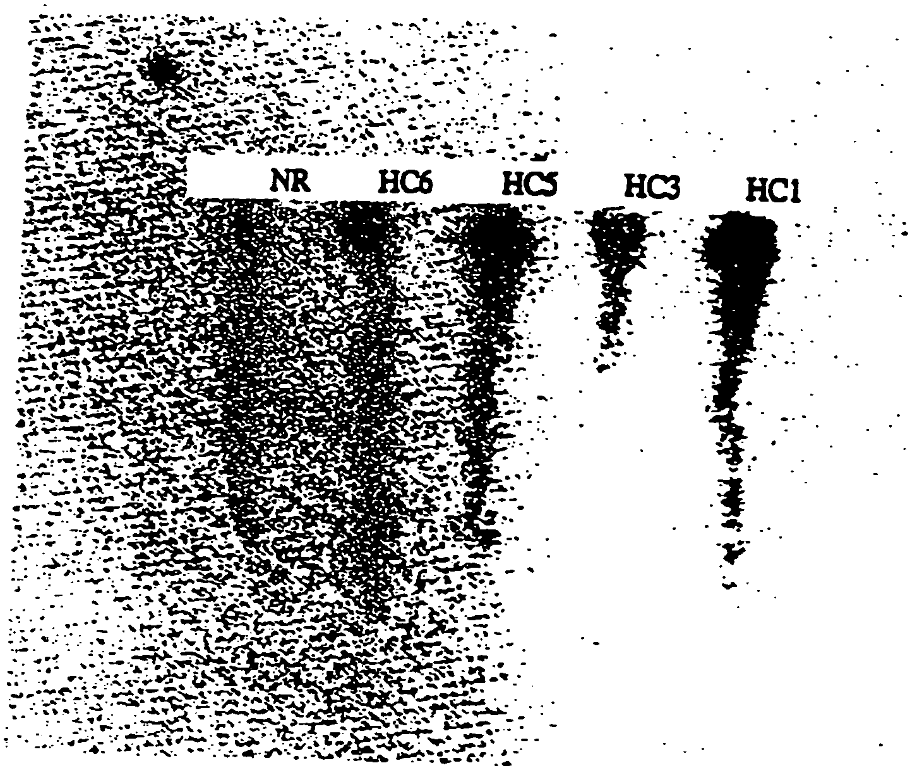


Fig. 3



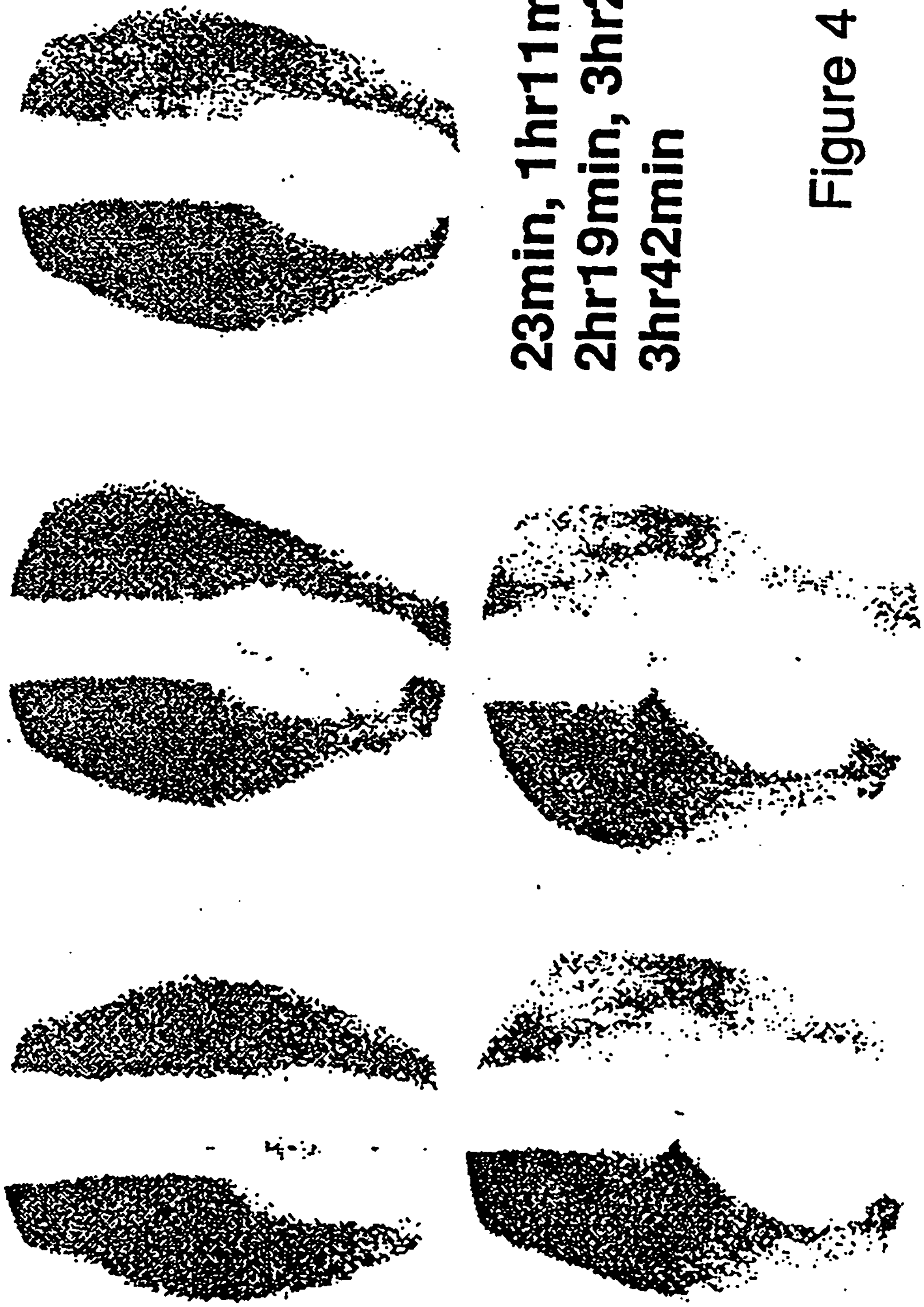



Figure 4

International Application No

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K49/02; A61K43/00

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | A61K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| P,X | WO,A,9 213 572 (DIATECH, INC) 20 August 1992 cited in the application see the whole document --- | 1-4,7-15 |
| X | TETRAHEDRON LETTERS vol. 30, no. 15, 1989, pages 1885 - 1888 H.K. MISRA ET AL. 'SYNTHESIS OF A NOVEL DIAMINODITHIOL LIGAND FOR LABELLING PROTEINS AND SMALL MOLECULES WITH TECHNETIUM-99M' cited in the application | 1-3,7-15 |
| A | see the whole document --- -/-- | 32-47, 50-52 |

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 22 JULY 1993 | ·? 08. 93 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | HOFF P.J. |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| X | WO,A,9 010 463 (NEORX CORPORATION) 20 September 1990 see abstract see page 6, line 29 - page 18, line 32 see page 26, line 3 - page 27, line 27 see page 29, line 33 - page 30, line 10; claims 1-17,29-33,72-75; examples 1-5 | 1-4, 7-15,50 |
| X | WO,A,8 910 759 (MALLINCKRODT, INC.) 16 November 1989 see abstract see page 13, line 4 - line 26; claims | 1-3, 7-15, 32-34, 38-47,50 |
| X | EP,A,0 188 256 (NEORX) 23 July 1986 cited in the application see the whole document | 1-3, 7-15,50 |
| A | INORGANIC CHEMISTRY vol. 29, no. 16, 1990, pages 2948 - 2951 N. BRYSON ET AL. 'PROTECTING GROUPS IN THE PREPARATION OF THIOLATE COMPLEXES OF TECHNETIUM' cited in the application see the whole document | 16-20, 23-31, 48-49 |
| A | BIOCONJUGATE CHEMISTRY vol. 1, no. 1, 1990, pages 132 - 137 K.E. BAIDOO ET AL. 'SYNTHESIS OF A DIAMINEDITHIOL BIFUNCTIONAL CHELATING AGENT FOR INCORPORATION OF TECHNETIUM-99M INTO BIOMOLECULES' cited in the application see the whole document | 32-34 |
| A | EP,A,0 403 243 (MERCK FROSST CANADA INC.) 19 December 1990 cited in the application see abstract see page 4, line 25 - line 35; claims | 32-34 |
| A | EP,A,0 174 853 (MALLINCKRODT INC.) 19 March 1986 see abstract; claims | 5-6, 21-22, 36-37 |

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| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| A | WO,A,9 116 919 (NEW ENGLAND DEACONESS HOSPITAL CORPORATION)<br>14 November 1991<br>see abstract<br>----- | 1-52 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303687
SA 73725

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 22/07/93
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| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9213572 | 20-08-92 | AU-A- | 1411792 | 07-09-92 |
| WO-A-9010463 | 20-09-90 | US-A- | 4986979 | 22-01-91 |
| | | EP-A- | 0463116 | 02-01-92 |
| | | JP-T- | 4504129 | 23-07-92 |
| WO-A-8910759 | 16-11-89 | AU-A- | 3778989 | 29-11-89 |
| | | EP-A- | 0381713 | 16-08-90 |
| EP-A-0188256 | 23-07-86 | DE-A- | 3680924 | 26-09-91 |
| | | JP-A- | 61225163 | 06-10-86 |
| | | US-A- | 4897255 | 30-01-90 |
| | | US-A- | 5175343 | 29-12-92 |
| | | US-A- | 5037630 | 06-08-91 |
| | | US-A- | 5120526 | 09-06-92 |
| EP-A-0403243 | 19-12-90 | CA-A- | 2019035 | 16-12-90 |
| | | JP-A- | 3081295 | 05-04-91 |
| EP-A-0174853 | 19-03-86 | US-A- | 4837003 | 06-06-89 |
| | | AU-B- | 595164 | 29-03-90 |
| | | AU-A- | 4737685 | 20-03-86 |
| | | CA-A- | 1276878 | 27-11-90 |
| | | EP-A- | 0350972 | 17-01-90 |
| | | EP-A- | 0397213 | 14-11-90 |
| | | JP-A- | 61072723 | 14-04-86 |
| WO-A-9116919 | 14-11-91 | AU-A- | 7992891 | 27-11-91 |



For more details about this annex : see Official Journal of the European Patent Office, No. 12/82